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The Regulation of Carbamoyl Phosphate Synthase Activity in Rat Liver Mitochondria

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1. The rate at which isolated rat liver mitochondria synthesized citrulline with NH_4Cl as nitrogen source was markedly dependent on the protein content of the diet. 2. Citrulline synthesis was not rate-limited by substrate concentration, substrate transport or ornithine transcarbamoylase activity under the conditions used. 3. The intramitochondrial content of an activator of carbamoyl phosphate synthase, assumed to be *N*-acetylglutamate, varied markedly with dietary protein content. The variation in the concentration of this activator was sufficient to account for the observed variation in the rates of citrulline synthesis if this synthesis were rate-limited by the activity of carbamoyl phosphate synthase. 4. The rates of urea formation from NH_4Cl as nitrogen source in isolated liver cells showed variations in response to diet that closely paralleled the variations in the rates of citrulline synthesis observed in isolated mitochondria. 5. These results are consistent with the postulate that when NH_4Cl plus ornithine are present in an excess, the rate of urea synthesis is regulated at the level of carbamoyl phosphate synthase activity.

Krebs *et al.* (1973*a*,*b*) have discussed in detail factors that may regulate the synthesis of urea in mammalian liver. In the urea cycle, the enzyme carbamoyl phosphate synthase (EC 2.7.2.5) is unique in its absolute requirement for the presence of an organic activator. This enzyme is localized in the mitochondrial matrix, and is totally inactive in the absence of *N*-acetylglutamate (Cohen & Sallach, 1961). Studies on the localization and biosynthesis of *N*-acetylglutamate together with variations in the tissue content of this compound in response to diet suggest that there may be conditions under which urea synthesis is regulated by carbamoyl phosphate synthase activity (Shigesada & Tatibana, 1971*a*,*b*). It is the purpose of the present investigation to identify one set of conditions under which such a regulation of urea synthesis may occur.

The starting point for the present investigation was the finding (McGivan *et al.*, 1974) that the rate at which isolated rat liver mitochondria synthesize citrulline is very dependent on the protein content of the diet on which the animals were fed. In the present paper, this work is extended to show that mitochondrial citrulline synthesis with NH_4Cl as nitrogen source is rate-limited by the intramitochondrial concentration of an activator of carbamoyl phosphate synthase. Experiments using isolated liver cells are consistent with the postulate that, under the conditions used, the conversion of NH_3 into urea is rate-limited by the same factor.

Experimental

Animals, diets and isolation of liver mitochondria and liver cells

Rats weighing 200–250g were maintained for 3 days on a standard diet (diet 41B; Oxoid Ltd., London S.E.1, U.K.), on boiled egg white or on 20% (w/v) glucose as originally described by Krebs (1972). The effectiveness of these diets in inducing changes in enzyme activity was monitored by routine assay of ornithine–2-oxoglutarate aminotransferase (EC 2.6.1.13) in sonicated mitochondria. The activity of this enzyme changes markedly in response to variations in diet (Krebs, 1972; McGivan *et al.*, 1974). The rats were killed on the morning of the fourth day after the introduction of the appropriate diet. Liver mitochondria were prepared as described by Chappell & Hansford (1972), except that sucrose in the isolation medium was replaced by 0.3M-mannitol, since sucrose interferes with the determination of citrulline. Liver cells were isolated by a modification of the method of Berry & Friend (1969) as described previously (Mendes-Mourão *et al.*, 1975) but with the omission of hyaluronidase.

Preparation of sonicated extracts of mitochondria and assay of intramitochondrial enzymes

The methods of preparation of sonicated extracts of mitochondria and of the assay of carbamoyl phos-

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phate synthase, ornithine transcarbamoylase (EC 2.1.3.3), ornithine–2-oxoglutarate aminotransferase and glutamate dehydrogenase (EC 1.4.1.2) in such extracts has been described (McGivan *et al.*, 1974). For the measurement of *N*-acetylglutamate synthesis, the mitochondrial extract was incubated with 10mM-acetyl phosphate, 0.5mM-CoA, 10mM-glutamate, 1mM-arginine plus phosphotransacetylase (EC 2.3.1.8) (20μg/ml) at pH7.2 and 37°C. The final protein concentration was 4–5mg/ml. Samples were withdrawn at zero time and after 10, 20 and 30min, deproteinized with HCl (0.3M final concentration) and assayed for *N*-acetylglutamate as described below.

N-Acetylglutamate hydrolysis was assayed as follows: mitochondrial sonicated extract (4–5mg/ml final concentration) was incubated with 0.1mM-*N*-acetylglutamate at pH7.2 and 37°C. Samples (1ml) were deproteinized at zero time and after 10, 20 and 30min and assayed for *N*-acetylglutamate as described below.

Assay of N-acetylglutamate in protein-free extracts of mitochondria

This compound was assayed by an indirect method based on measurement of the activation of carbamoyl phosphate synthase by a protein-free extract of mitochondria. Although this assay is very sensitive it has the disadvantage that it is not specific for *N*-acetylglutamate and will detect any activator of carbamoyl phosphate synthase. However, *N*-acetylglutamate has been shown to occur in liver mitochondria (Shigesada & Tatibana, 1971*a*), and no other compound has been shown to activate carbamoyl phosphate synthase to anything like the same extent as does *N*-acetylglutamate.

A sonicated extract of mitochondria serves as the source of carbamoyl phosphate synthase. The extract is incubated with NH_4Cl, ATP, ornithine, $KH^{14}CO_3$ and the extract containing *N*-acetylglutamate. Under these conditions, the rate at which [^{14}C]bicarbonate is converted into [^{14}C]carbamoyl phosphate is controlled by the concentration of *N*-acetylglutamate. The presence in the mitochondrial extract of ornithine transcarbamoylase causes the immediate reaction of [^{14}C]carbamoyl phosphate to form [^{14}C]citrulline. The activity of this latter enzyme in the sonicated extract is at least 20-fold in excess of that of carbamoyl phosphate synthase. The rate of conversion of [^{14}C]-bicarbonate into [^{14}C]citrulline thus depends solely on the concentration of *N*-acetylglutamate, and is negligible in the absence of this compound. On acidification of the incubation mixture, followed by removal of the denatured protein and heating of the extract, the excess of $KH^{14}CO_3$ is decomposed to $^{14}CO_2$ which is evolved. Similarly the radioactivity from any [^{14}C]carbamoyl phosphate is evolved as $^{14}CO_2$. The radioactivity remaining in the extract is assayed, and this represents [^{14}C]citrulline. A similar principle for the measurement of [^{14}C]citrulline in the presence of $KH^{14}CO_3$ or [^{14}C]carbamoyl phosphate has been used by Natale & Tremblay (1974).

The details of the method are as follows. Mitochondria were used without prior incubation and within 30min of preparation. Samples of the mitochondrial suspension were diluted with cold preparation medium (Chappell & Hansford, 1972) to give suspensions with protein concentrations in the range 5–15mg of protein/ml. These suspensions were deproteinized by the addition of 3M-HCl to give a final concentration of 0.3M-HCl. The precipitated protein was removed by centrifugation for 2min in an Eppendorf Zentrifuge model 3200. The extracts were then neutralized with KOH to give a final pH of between 7.1 and 7.2; this was verified by using a pH-meter. Carbamoyl phosphate synthase activity is very pH-dependent and is also inhibited by perchlorate anions. Hence $HClO_4$ could not be used to deproteinize the mitochondria.

Samples (0.6ml) of the neutralized extracts were incubated with 20mM-ornithine, 20mM-NH_4Cl, 5mM-ATP, 15mM-$MgCl_2$, 10mM-$KHCO_3$ and 0.5μCi of $KH^{14}CO_3$/ml at pH7.2 and 37°C in 1ml, which also contained a sonicated extract of mitochondria (3mg/ml final concn.). The rate of citrulline synthesis in this incubation was linear with time over a 20min period. A sample was withdrawn at zero time and deproteinized to obtain a value for residual radioactivity caused by compounds other than citrulline. After precisely 10min, the incubations were terminated by the addition of $HClO_4$ (5%, w/v, final concn.). After centrifugation, 0.5ml samples of the extracts were heated for 5min in a boiling-water bath, cooled, added to 15ml of scintillator solution and assayed for radioactivity in a Nuclear–Chicago Isocap/300 Liquid-Scintillation System, incorporating corrections to allow for quenching. The scintillator solution used contained in 1 litre: 600ml of toluene, 400ml of 2-methoxyethanol and 6g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole.

The assay was calibrated by replacing the mitochondrial suspension with a standard solution of *N*-acetylglutamate which was put through the same procedure of acidification and neutralization, and several such standards with appropriate blanks were measured with each batch of mitochondrial extracts. That the radioactivity in the final extract was due only to [^{14}C]citrulline was confirmed by the fact that no increase in acid-stable radioactivity occurred in the 10min incubation period if ornithine was omitted.

The radioactivity (d.p.m.) found in the final solution was proportional to the amount of *N*-acetylglutamate in the original extract over the range 0–50nmol/ml. The sensitivity of the assay was normally in the range 50–100d.p.m./nmol of *N*-acetylglutamate.

Incubation of isolated liver cells

Liver cells (approx. 3 mg of protein/ml, final concn.) were suspended in Ca^{2+}-containing Krebs–Henseleit medium (Krebs & Henseleit, 1932) with added 10mM-NH_4Cl, 10mM-sodium lactate, 5mM-ornithine and 2.5% (w/v) bovine albumin at 37°C. The albumin had been dialysed for 48h against Krebs–Henseleit medium, but had not been defatted. The incubation medium was continuously gassed with O_2+CO_2 (95:5) with shaking. The pH of the incubation remained at 7.4 throughout. Samples were taken at zero time and after 20, 30 and 40min and deproteinized by the addition of $HClO_4$ (5%, w/v, final concn.). The samples were neutralized with KOH, cooled and assayed for metabolites after the removal of $KClO_4$ by centrifugation.

Assay of metabolites

NH_3 was assayed as described by Kirsten *et al.* (1963), and urea was determined in the same sample by measuring the release of NH_3 consequent on the addition of urease (0.1 mg/ml). Glutamate was determined enzymically (Bernt & Bergmeyer, 1965). Citrulline was assayed by the method of Archibald (1944). Glutamine was determined as follows: 0.5ml of neutralized extract was added to 0.7ml of 0.1M-sodium acetate buffer, pH5.0. Glutaminase (0.1 mg/ml final concn.) was added and the solution was incubated at 20°C for 30min. Then 1 ml of glycine/hydrazine buffer (0.5M-hydrazine plus 0.4M-glycine, pH9.5) was added, together with NAD^+ (1.3mg/ml final concn.) and glutamate dehydrogenase (0.2mg/ml final concn.) and the increase in E_{340} was measured after a further 30min. This method measures total glutamate plus glutamine. Glutamate was determined separately as described above. Mitochondrial and liver-cell protein was determined by a biuret method (Gornall *et al.*, 1949).

Enzymes, nucleotides and carbamoyl phosphate were purchased from C. F. Boehringer und Söhne G.m.b.H., Mannheim, Germany. *N*-Acetyl-L-glutamic acid, glutaminase and bovine albumin (fraction V) were purchased from the Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Collagenase for the preparation of liver cells was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Results

Nature of the rate-limiting reaction in the synthesis of citrulline from NH_4Cl as nitrogen source in rat liver mitochondria

Isolated liver mitochondria synthesize citrulline when incubated with ornithine, bicarbonate, NH_4Cl and a source of ATP (Charles *et al.*, 1967). This synthesis involves the initial formation of carbamoyl phosphate from bicarbonate, ATP and NH_3 catalysed by carbamoyl phosphate synthase, the transport of ornithine into the mitochondrial matrix space, the formation of citrulline from ornithine plus carbamoyl phosphate catalysed by ornithine transcarbamoylase and finally the transport of citrulline out of the mitochondria into the surrounding medium. This sequence of reactions constitutes the first part of the urea cycle. In the presence of saturating concentrations of substrates it is in principle possible for the synthesis of citrulline from NH_4Cl to be rate-limited by the activity of either of the two enzymes involved or by the transport of ornithine or citrulline across the mitochondrial membranes.

Table 1 defines the optimum conditions for citrulline synthesis when substrates were present at saturating concentrations. The rate of citrulline synthesis depended on the mechanism by which ATP was supplied. As shown by Graafmans *et al.* (1968), exogenously added ATP is a very poor substrate for citrulline synthesis unless an uncoupler of oxidative phosphorylation is present to facilitate ATP transport across the inner membrane. Oligomycin must also be present to prevent ATP hydrolysis by the mitochondrial adenosine triphosphatase. Even in the presence of these two reagents, the rate of synthesis of citrulline was lower than when ATP was generated by oxidative phosphorylation with succinate as respiratory substrate. The addition of exogenous ADP did not further increase the rate.

Under the optimum conditions defined above, when

Table 1. *Optimum conditions for mitochondrial citrulline synthesis*

Mitochondria (6–7mg of protein/ml) isolated from rats fed on a normal diet were incubated in a medium containing 80mM-KCl, 20mM-Tris/HCl, 20mM-$KHCO_3$, 10mM-ornithine/HCl, 10mM-NH_4Cl, 5mM-potassium phosphate, 10μg of rotenone/ml and further additions as shown at 30°C and pH7.2. The medium had been previously gassed with O_2+CO_2 (95:5). The suspension was incubated with rapid shaking. Samples were withdrawn at zero time and after 2, 4 and 6min, deproteinized and assayed for citrulline. The synthesis of citrulline was approximately linear with time over this interval.

Additions	Rate of citrulline synthesis (nmol/min per mg)
None	0.4
ATP (5mM)	3.2
ATP (5mM)+oligomycin (10μg/ml)	3.3
ATP (5mM)+carbonyl cyanide phenylhydrazone (1μM)	0.3
ATP (5mM)+carbonyl cyanide phenylhydrazone (1μM)+oligomycin (10μg/ml)	10.1
Succinate (10mM)	14.1
Succinate (10mM)+ADP (2mM)	12.2

Table 2. *Variation in the rate of mitochondrial citrulline synthesis and in enzyme activity as a function of the protein content of the diet*

Mitochondrial citrulline synthesis was measured as described in the legend to Table 1, with 10mM-succinate as respiratory substrate. Carbamoyl phosphate synthase activity was measured in the presence of 5mM-*N*-acetylglutamate. Each value quoted represents the mean±S.E.M. of observations made on separate mitochondrial preparations from at least four different groups of animals. * P <0.001 versus standard diet by Student's t test.

Diet	Rate of reaction (nmol/min per mg of protein)		
	Citrulline synthesis by intact mitochondria	Carbamoyl phosphate synthase in sonicated mitochondria†	Ornithine transcarbamoylase in sonicated mitochondria†
Standard diet	15.3±1.1	27.8±5.5	690±150
Egg white	35.5±2.9*	33.8±6.4	1220±238
Glucose (20%, w/v)	2.3±0.5*	21.4±1.3	697± 49

† Results from McGivan *et al.* (1974).

substrates were present in an excess and ATP was supplied by oxidative phosphorylation, the rate of citrulline synthesis by isolated mitochondria depended on the protein content of the diet of the animals from which the mitochondria were isolated (Table 2). Citrulline synthesis was considerably increased in mitochondria from rats fed on a high-protein diet and decreased in rats fed on glucose. Table 2 shows the maximum activities of carbamoyl phosphate synthase (assayed in the presence of 5mM-*N*-acetylglutamate) and ornithine transcarbamoylase in mitochondria where permeability barriers had been removed by sonication. These results suggest that the activity of these two enzymes was in general greater than the rate at which citrulline synthesis occurred, and that the relatively small adaptive changes in enzyme activity were inadequate to account for the variations in the rate of citrulline synthesis observed as a function of diet.

The possibility that citrulline synthesis was limited by the rate of transport of ornithine or citrulline was investigated. Direct measurement of ornithine and citrulline transport is not practicable at the present time, and the mechanism by which these substrates are transported has not been satisfactorily elucidated. Accordingly, an indirect measure of transport was used. Mitochondria were incubated with ornithine plus 10mM-carbamoyl phosphate. Carbamoyl phosphate has been shown to penetrate the mitochondrial membrane (Natale & Tremblay, 1974). Fig. 1 shows that the rate of citrulline synthesis obtained in the presence of high ornithine concentrations was approx. 60nmol/min per mg of protein. The citrulline formed must be immediately transported out of the mitochondria. If this were not the case, the intramitochondrial concentration of citrulline would become very large and the mitochondria would burst as a result of osmotic pressure. It follows that both ornithine and citrulline must be translocated at a minimum rate of 60nmol/min per mg under these

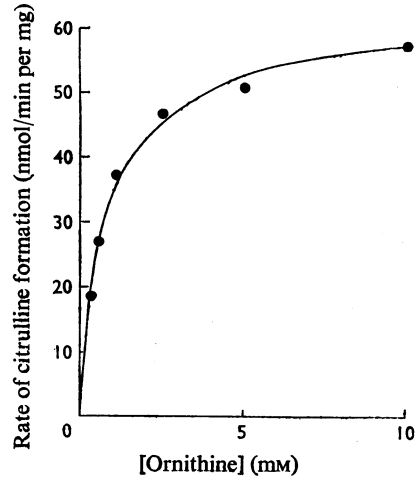

Fig. 1. *Citrulline formation from ornithine plus carbamoyl phosphate in intact mitochondria*

Mitochondria from rats fed on a standard diet were incubated at 30°C and pH7.2 in a medium containing 0.12M-KCl, 20mM-Tris/HCl, 10μg of rotenone/ml, 10mM-carbamoyl phosphate and various concentrations of ornithine as shown. The rate of citrulline synthesis was obtained from the analyses of samples withdrawn after 1, 2 and 3min.

conditions. The rate of citrulline synthesis from ornithine plus carbamoyl phosphate was not decreased in mitochondria from glucose-fed rats (results not shown), a fact that suggests that the transport of ornithine and citrulline was not rate-limiting for citrulline synthesis from NH_4Cl as nitrogen source.

It appears that the rate-limitation may be at the level of activation of carbamoyl phosphate synthesis by the intramitochondrial concentration of *N*-acetylglutamate. Exogenously added *N*-acetylglutamate

did not stimulate citrulline synthesis in mitochondria from glucose-fed rats (results not shown). However, it is likely that this compound does not readily penetrate the inner mitochondrial membrane (Charles *et al.*, 1967).

Intramitochondrial content of an activator of carbamoyl phosphate synthase

Support for the postulate that citrulline synthesis under the conditions used was limited by the rate of the carbamoyl phosphate synthase reaction was obtained by measurement of the intramitochondrial concentration of an activator of this enzyme as a function of diet. Table 3 shows the intramitochondrial content of an activator of this enzyme expressed in terms of the amount of authentic *N*-acetylglutamate which produced an equivalent activation. There was a marked variation in the intramitochondrial content of this compound as a function of the protein content of the diet.

Fig. 2 shows the characteristics of the activation of carbamoyl phosphate synthase by *N*-acetylglutamate in a sonicated extract of mitochondria. Half-maximum activation occurred at a concentration of approx. 0.2mM-*N*-acetylglutamate. In four separate preparations, the concentration of *N*-acetylglutamate required for half-maximum activation was 0.18±0.03mM. It may be noted that this value is much lower than that (1mM) found for carbamoyl phosphate synthase isolated from frog liver (Fahien & Cohen, 1964).

On the assumption that the activator of carbamoyl phosphate synthase measured in Table 3 is in fact *N*-acetylglutamate, the following points may be noted. The absolute concentration of *N*-acetylglutamate cannot be determined directly from the value in Table 3, since an unknown amount of this compound is protein-bound and not available to the enzyme. If the arbitrary assumption is made that the amount of *N*-acetylglutamate that is protein-bound is about 0.2nmol/mg of protein and the intramitochondrial volume is taken to be 1μl/mg of protein (see e.g. Halestrap & Denton, 1974), the concentrations of *N*-acetylglutamate present in the matrix in the case of standard, egg white and glucose diets may be calculated to be 0.4, 1.1 and 0.06mM respectively. The concentration of *N*-acetylglutamate required for half-maximum activation of carbamoyl phosphate synthase in the mitochondrial matrix may be assumed to be about 0.2mM (c.f. Fig. 2). These estimated concentrations of *N*-acetylglutamate would therefore allow carbamoyl phosphate synthesis to occur at rates which are 67, 85 and 23% respectively, of the maximum velocity of carbamoyl phosphate synthase.

By using the values of carbamoyl phosphate synthase activity shown in Table 2, the rates of carbamoyl phosphate synthesis predicted on the above assumptions are 18.6±3.7, 28.7±5.6 and 4.9±0.3nmol/min per mg of protein, for mitochondria isolated from rats fed on standard, egg white and glucose diets respectively. These values are similar to the respective rates of citrulline synthesis reported in Table 2.

To this extent the results support the postulate that mitochondrial citrulline synthesis regulated at the level of carbamoyl phosphate synthase activity. However, at present there is no basis on which the intramitochondrial concentration of free *N*-acetylglutamate can be even approximately estimated.

The activity of *N*-acetylglutamate synthetase was determined in sonicated extracts of mitochondria in

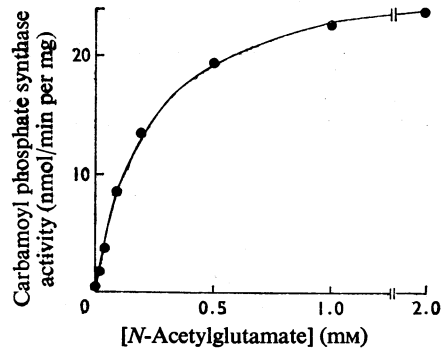

Fig. 2. *Dependence of carbamoyl phosphate synthase activity on added N-acetylglutamate*

A sonicated extract of mitochondria (2mg of protein/ml final concn.) was incubated in a medium containing 0.08M-KCl, 20mM-Tris/HCl, 20mM-$KHCO_3$, 20mM-ornithine, 20mM-NH_4Cl, 10mM-ATP, 15mM-$MgCl_2$, 5μg of oligomycin/ml plus various concentrations of *N*-acetylglutamate at pH7.2 and 30°C. The rate of citrulline synthesis was assayed by taking samples over the first 10min of the reaction, when the progress curve was linear. The rate of synthesis of carbamoyl phosphate was taken as equal to that of the formation of citrulline measured (see the Experimental section).

Table 3. *Intramitochondrial content of an activator of carbamoyl phosphate synthase*

Details of the assay system are given in the Experimental section. The results are given as the means±S.E.M. of separate mitochondrial preparations from the numbers of groups of animals shown in parentheses. ** $P<0.05$ versus standard diet.

Diet	Equivalent content of *N*-acetylglutamate (nmol/mg of protein)
Standard diet	0.59±0.08 (9)
Egg white	1.30±0.32 (5)**
Glucose (20%, w/v)	0.26±0.05 (4)**

Table 4. *Rates of NH_3 utilization and metabolite formation in isolated rat liver cells*

Liver cells were incubated with NH_4Cl, lactate and ornithine as described in the Experimental section. The rates were evaluated from progress curves that were linear with time over 40 min. Rates are expressed as nmol or ng-atoms of metabolite/min per mg of cell protein throughout. Each value is the mean ± S.D. of a minimum of eight measurements, involving at least two cell preparations. The rates obtained are somewhat lower than those reported by Krebs *et al.* (1974) for similar experiments.

Diet	Metabolite formation: Urea	Glutamate	Glutamine	NH_3 disappearance	N atoms appearing in urea, glutamate and glutamine
Standard diet	2.1 ± 0.2	0.14 ± 0.02	0.16 ± 0.02	4.22 ± 0.35	4.66 ± 0.4
Egg white	8.1 ± 0.5	0.20 ± 0.01	0.23 ± 0.05	14.3 ± 1.3	16.86 ± 1.0
Glucose (20%)	1.4 ± 0.4	0.30 ± 0.01	0.89 ± 0.21	5.3 ± 0.25	4.88 ± 0.9

the presence of 1 mM-arginine, which has been reported to activate this enzyme (Shigesada & Tatibana, 1971*b*). In rats fed on a standard diet, *N*-acetylglutamate was synthesized at a rate of 0.25 ± 0.05 nmol/min per mg (mean ± S.E.M. for four preparations). With mitochondria from glucose-fed rats, the rate of *N*-acetylglutamate synthesis was less than 0.01 nmol/min per mg. No *N*-acetylglutamate hydrolase activity was detectable in preparations of sonicated mitochondria from either source. These results provide preliminary evidence that the intramitochondrial concentration of *N*-acetylglutamate may be controlled, in part, by the activity of *N*-acetylglutamate synthetase.

Conversion of NH_3 into urea in isolated liver cells

Liver cells were prepared from rats that had been fed on different diets. The cells were incubated in the presence of NH_4Cl, lactate and ornithine at concentrations that were not limiting for urea synthesis. Table 4 shows that the ammonia nitrogen that disappeared was recovered quantitatively in the form of urea, glutamate and glutamine, except in cells prepared from rats fed on a high-protein diet, where the nitrogen recovered in urea, glutamate plus glutamine slightly exceeded the value of NH_3 disappearance. This was indicative of some metabolism of endogenous substrates.

The rates of both NH_3 disappearance and urea formation increased markedly with increasing protein content of the diet (Table 4). In cells from glucose-fed rats, the rate of NH_3 utilization was relatively low; in this case the rates of synthesis of urea and of glutamate plus glutamine were comparable. The rates of urea synthesis in liver cells showed the same adaptive charges in response to diet as did the rates of citrulline synthesis from NH_4Cl in isolated mitochondria. Further, on the assumption that 1 g wet weight of liver is equivalent to 40 mg of mitochondrial protein or to 140 mg of cell protein, the rates of urea synthesis in cells, expressed as μmol/min per g wet wt. of liver, are comparable (to within a factor of 2) with the rates of mitochondrial citrulline synthesis expressed in the same units.

In the formation of one molecule of urea from NH_4Cl, one NH_3 molecule is converted into glutamate by the action of glutamate dehydrogenase and then into aspartate by the action of glutamate–oxaloacetate transaminase; another NH_3 molecule is converted into carbamoyl phosphate and hence into citrulline. It has been shown (McGivan *et al.*, 1974) that the activities of glutamate–oxaloacetate transaminase and of glutamate dehydrogenase (in the direction of glutamate synthesis) in mitochondria are high and are little affected by the protein content of the diet. Further, it has been reported that the intracellular concentration of citrulline is very low under normal conditions (see Krebs *et al.*, 1973*b*). The considerations, together with the results shown in Table 4, are consistent with the postulate that the synthesis of urea by liver cells in the presence of saturating concentrations of NH_4Cl and ornithine is regulated by the activity of carbamoyl phosphate synthase.

Discussion

The present study provides evidence for a limitation of the rate of mitochondrial citrulline synthesis by the intramitochondrial concentration of an activator of carbamoyl phosphate synthase, presumably *N*-acetylglutamate. Evidence is also presented that is consistent with the postulate that urea synthesis from NH_3 is rate-limited by the same factor. *N*-Acetylglutamate in the rat is localized mainly in tissues that catalyse ureogenesis. The hepatic content of this compound increases with the protein content of the diet and, in liver, this compound is found very largely in the mitochondrial fraction (Shigesada & Tatibana, 1971*a*). *N*-Acetylglutamate synthetase is localized in the mitochondria and is activated by low concentrations of arginine (Shigesada & Tatibana, 1971*b*). On the basis of these and other findings, it has been assumed that *N*-acetylglutamate may have a regulatory role in urea synthesis (see Krebs *et al.*,

1973*a*,*b*). However, this has not been previously demonstrated. It is suggested that the experiments described above define one set of conditions where carbamoyl phosphate synthase is rate-limiting, namely when NH_4Cl, ornithine and ATP are present in an excess. It remains to be demonstrated whether the activity of this enzyme is rate-limiting when NH_3 is provided continuously by the deamination of amino acids, or when the ornithine concentration is low.

The activity of *N*-acetylglutamate synthetase in sonicated mitochondria is very much lower than the activities of most other enzymes in the mitochondrial matrix. The activity of *N*-acetylglutamate hydrolase is also very low. *N*-Acetylglutamate does not penetrate the mitochondrial membrane with any facility, since exogenously added *N*-acetylglutamate did not stimulate mitochondrial citrulline synthesis. It therefore appears that changes in the intramitochondrial concentration of *N*-acetylglutamate would occur over relatively long time-intervals, particularly if such changes were brought about by the long-term induction or repression of *N*-acetylglutamate synthetase in response to dietary changes. Regulation of urea synthesis at the level of carbamoyl phosphate synthase activity should therefore be regarded as an example of long-term rather than acute metabolic control.

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